

FBS07- Microscopic Examination of Spermatozoa by Christmas Tree Stain

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1. Scope

- 1.1. This procedure is used to confirm the presence of spermatozoa/semen.

2. Background

- 2.1. The microscopic identification of spermatozoa is a method of confirming the presence of semen in an evidentiary stain. Spermatozoa are identified by either the presence of intact sperm cells displaying a head, mid-piece and tail, or sperm heads showing an acrosomal cap.
- 2.2. The microscopic examination can be enhanced by staining the slide using a differential stain known as “Christmas Tree Stain” which consists of two dyes: Nuclear Fast Red and picroindigocarmine. Sperm heads are usually well differentiated (red) with the acrosome staining significantly less densely (pink) than the distal region of the head. Nuclei inside epithelial cells appear pink to purple in color. Sperm tails and epithelial membranes are stained green by the picroindigocarmine.

3. Safety

- 3.1. Wear personal protective equipment (e.g., lab coat, gloves, mask, eye protection), when carrying out standard operating procedures (SOPs).
- 3.2. Read Safety Data Sheets (SDSs) to determine the safety hazards for chemicals and reagents used in the SOPs.

4. Materials Required

- 4.1. TE (Tris EDTA) buffer or autoclaved deionized water (diH₂O) (FBR06)
- 4.2. Nuclear Fast Red Dye or SERI R540 Christmas Tree Stain A
- 4.3. Picroindigocarmine Solution or SERI R540 Christmas Tree Stain B
- 4.4. Millipore H₂O
- 4.5. 95% Ethanol
- 4.6. Slides
- 4.7. Coverslips
- 4.8. 2.0 mL microcentrifuge tubes (optional)
- 4.9. Microscope

5. Standards and Controls

- 5.1. It is not necessary to prepare Positive and Negative Control slides for this procedure. **Optional:** The slide(s) created by the quality control procedure (FBQ21) may be used as Positive reference slide(s).
- 5.2. If a sample is retained for future DNA testing the appropriate Negative Control(s) must accompany the sample.

6. Procedures

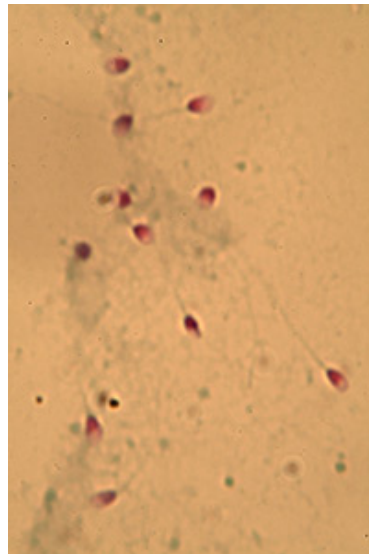
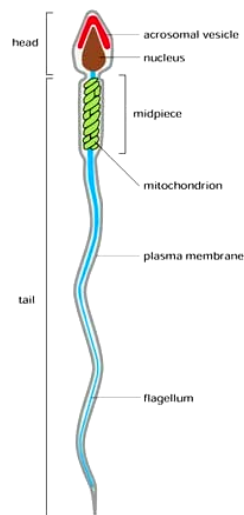
- 6.1. Follow all the steps if preparing a slide from a cutting. If preparing a slide from a p30 sample, begin with step 6.4.1. For smear slides and differential extraction samples, begin with step 6.7.
- 6.2. Following the chart below, add an appropriate volume of TE to sample(s) followed by the appropriate volume of TE for the Negative Control (TE only), if applicable. If a Negative Control is created, it must follow the same procedure below as its associated sample(s) (i.e., same lot of reagent(s) and number of washes).

Dimensions of Cutting (cm)	TE Volume (µL)
0.5 x 0.5	250
0.7 x 0.7	500
1.0 x 1.0	1000
1/4 of swab	1000

- 6.2.1. Allow the sample(s) to incubate at room temperature for at least two hours. If incubating overnight, place the sample(s) in the refrigerator at 4°C. Samples may be placed on an orbital shaker during the incubation time. If refrigerated, be sure to allow the samples to come to room temperature prior to proceeding to the next step. Following incubation, vortex briefly and quick-spin down.
- 6.3. Transfer the sample to a filterless basket in a 2.0 mL microcentrifuge tube and spin the sample tube(s) for 3 minutes at maximum speed. The substrate may be retained for future DNA testing.
- 6.4. Remove the supernatant until approximately 50 µL is left in the tube, taking care to not disturb the pellet.
- 6.4.1. **Optional:** Depending on the nature of the sample, the sperm pellet may be washed by re-suspending in 500-1000 µL of TE Buffer or diH₂O. (If the TE Buffer or diH₂O lot is different from the previously used lot, then an additional Negative Control will be created and processed alongside the associated sample if that associated sample proceeds to DNA testing.) Vortex and spin the sample(s) in a microcentrifuge for 3-5 minutes at maximum velocity. Remove and discard the supernatant, being careful not to disturb the sperm pellet (up to 50 µL may be left in the tube). Repeat this washing step an additional 2 times for a total of 3 washes of the sperm pellet. After the final spin, remove and discard all but approximately 50 µL of the supernatant, taking care not to disturb the sperm pellet.
- 6.5. Properly label a slide with sample identifier.
- 6.6. Re-suspend sperm pellet by agitating with a pipet tip. Pipet 4 µL of the re-suspended sperm pellet onto the slide. The remainder of the sample may be recombined with the substrate and stored at -20°C for DNA extraction.
- 6.6.1. If the sample (substrate, extract and/or pellet) will be used for differential extraction, then a Negative Control must be created and processed alongside the extract. The Negative Control(s) must be made using the same lot of reagent(s) (TE Buffer and/or diH₂O) used to make the extract and wash the sperm pellet. For more information regarding the

processing, analysis, and/or interpretation of the Negative Control, see the applicable SOPs.

- 6.7. Heat-fix cells to the microscope slide by conducting one of the following:
 - incubating in an oven (at 60°C for at least 1 hour)
 - incubating on a hot plate (at high for 20-30 minutes)
 - passing the slide (2-4 times) through the flame from a Bunsen Burner
- 6.8. Cover the stained area with Nuclear Fast Red Solution or Stain A.
- 6.9. Allow the slide to incubate at room temperature for at least 15 minutes.
- 6.10. Wash the slide gently with Millipore H₂O until it washes clear.
- 6.11. Cover the stain area with Picroindigocarmine stain or Stain B.
- 6.12. Wash the slide after 5 seconds with 95% ethanol until it washes clear.
- 6.13. Allow slide to air dry or dry on a hot plate.
- 6.14. Wet mount slide using Millipore H₂O and coverslip. Using a microscope, view the slide under 200-400x magnification. Nuclei inside epithelial cells appear pink to purple in color. Sperm tails and epithelial membranes are stained green. The sperm head is usually well differentiated with the acrosomal cap staining pink and the nuclear material staining red (see diagram below).



Note: Slides may be viewed with a light microscope with the option of using a phase contrast filter.

- 6.15. Observations of sperm cells, such as intact and/or sperm heads, will be noted in the applicable Sample Tracking and Control Solutions (STACS) documentation. Additionally, the presence of spermatozoa will be documented as follows:

- +4 More than one sperm observed in every examined field
- +3 Sperm cells observed without difficulty in at least >50% to 90% of examined fields
- +2 Sperm cells observed in >10% to 50% of examined fields
- +1 Sperm cells observed in less than 10% of all fields
- 1 One sperm observed on entire slide
- 0 No sperm

Note: If only a single spermatozoon is found on a slide, the coordinates of the spermatozoon location and slide orientation will be documented, in case the spermatozoon needs to be relocated.

7. Sampling

- 7.1. Not applicable

8. Calculations

- 8.1. Not applicable

9. Uncertainty of Measurement

- 9.1. Not applicable

10. Limitations

- 10.1. Insufficient sample quality, quantity, and/or heavy debris could limit the detection of spermatozoa.
- 10.2. Yeast cells stain red and may resemble a sperm head. However, the stain is uniform throughout the cell and extends into polyp-like structures, which are occasionally observed with yeast cells.
- 10.3. If only a single spermatozoon is found on a slide, a newly trained analyst (i.e., less than 3 months of sperm search experience) will have another qualified

analyst verify the spermatozoon. The verification will be noted in the casework documentation.

11. Documentation

11.1. Applicable STACS documentation

11.2. FBU Report of Examination

12. References

12.1. Serological Research Institute. Christmas Tree Stain R540 Informational Flyer, February 1999.

12.2. P30 Antigen Test for the Presence of Semen (FBS06)

12.3. Differential Organic DNA Extraction (FBS09)

12.4. Quality Control of Christmas Tree Stain Reagents (FBQ21)

12.5. Sterile Deionized Water (FBR06)

12.6. Forensic Biology Unit Quality Assurance Manual

12.7. EZ1 Advanced XL – DNA Extraction (FBS20)